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Bacterial inoculants affecting nickel uptake by *Alyssum murale* from low, moderate and high Ni soils

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Abstract

Metal hyperaccumulator plants like *Alyssum murale* have a remarkable ability to hyperaccumulate Ni from soils containing mostly insoluble Ni. We have shown some rhizobacteria increase the phytoavailability of Ni in soils, thus enhancing Ni accumulation by *A. murale*. Nine bacterial strains, originally isolated from the rhizosphere of *A. murale* grown in serpentine Ni-rich soil, were examined for their ability to solubilize Ni in different soils and for their effect on Ni uptake into *Alyssum. Microbacterium oxydans* AY509223; *Rhizobium galegae* AY509213; *Microbacterium oxydans* AY509219; *Clavibacter xyli* AY509236; *Acidovorax avenae* AY512827; *Microbacterium* a*rabinogalactanolyticum* AY509225; *M. oxydans* AY509222; *M. arabinogalactanolyticum* AY509226 and *M. oxydans* AY509221 were added to low, moderate and high Ni-contaminated soils. *M. oxydans* AY509223 significantly increased Ni extraction by 10 mM $Sr(NO_3)_2$ from the high and medium soils and had no effect on Ni extraction from the low Ni soils. The other eight bacterial isolates significantly increased Ni extraction from all soils. There were no significant effects of bacterial inoculation on fresh and dry weight of *A . murale* shoots grown in the low and high Ni soils compared to an unamended control. *M. oxydans* AY509223 significantly increased Ni uptake of *A. murale* grown in the low, medium, and high soils by 36.1%, 39.3%, and 27.7%, respectively, compared with uninoculated seeds. *M. oxydans* AY509223 increased foliar Ni from the same soils from 82.9, 261.3 and 2829.3 mg kg^{-1} to 129.7, 430.7, and 3914.3 mg kg^{-1}, respectively, compared with uninoculated controls. These results show that bacteria are important for Ni hyperaccumulation and could potentially be developed as an inoculum for enhancing uptake during commercial phytoremediation or phytomining of Ni.

Keywords: *Alyssum murale*; Bioavailability; Phytoextraction; Rhizobacteria; Serpentine soils

1. Introduction

Heavy metal contamination in soils is one of the world's major environmental problems, posing significant risks to human health as well as to ecosystems. Therefore, the development of a remediation strategy for metal-contaminated soils is necessary for environmental conservation and human health. Phytoremediation, using plants to remove metal pollutants from contaminated soils, is being developed as a new method for the remediation of contaminated land. This environmentally friendly, cost-effective and plant-based technology is expected to have significant economic, aesthetic, and technical advantages over traditional engineering techniques (Chaney, 1983; Baker et al., 1994; Glass, 2000; Susarla et al., 2002; Chaney et al., 2005). Phytoremediation uses hyperaccumulator plants that have the ability to accumulate very high metal concentrations from contaminated soils in their shoots (Baker and Brooks, 1989). Among the best-known hyperaccumulators, *Alyssum murale* is able to colonize serpentine soils and accumulate nickel in excess of 2% (W/W) of shoot dry-matter (Reeves and Baker, 2000).

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Heavy metals in soils are generally bound to organic and inorganic soil constituents, or alternatively, present as insoluble precipitates, a large proportion of metal contaminants are unavailable for root uptake by field-grown plants. Methods of increasing heavy metal contaminants' phytoavailability in soil and its transport to plant roots are vital to the success of phytoremediation in the field (Ernst, 1996; Kukier et al., 2004).

The rhizosphere provides a complex and dynamic microenvironment where microorganisms, in association with roots, form unique communities that have considerable potential for detoxification of hazardous waste compounds (Black et al., 1993; De-Souza et al., 1999). Microbial populations are known to affect trace metal mobility and availability to the plant, through release of chelators, acidification, and redox changes (Smith and Read, 1997; Abou-Shanab et al., 2003a). Improvement of the interactions between plants and beneficial rhizosphere microorganisms can enhance biomass production and tolerance of the plants to heavy metals, and are considered to be an important component of phytoremediation technologies (Wenzel and Jockwer, 1999; Glick, 2003).

Given the potential importance of bacteria in Ni uptake by plants, the goal of this work was to study whether nine different bacterial strains isolated from the rhizosphere of *A. murale* were able to increase Ni solubilization in low, moderate and Ni-rich soils and whether subsequent uptake of Ni by *A. murale* was affected.

2. Materials and methods

2.1. Soil sampling and preparation

Three soils were used in this study: (1) Christiana fine sandy loam (Beltsville, Maryland) soil, a low Ni soil; (2) Chrome silt loam soil from the Soldiers Delight Ecological Reservation, a moderate Ni-serpentine soil collected in Baltimore County, Maryland and (3) a Brockman variant gravelly silt loam, a Ni-rich serpentine soil collected near Cave Junction, Oregon. Soils were mixed in large containers and dried at room temperature. Rocks were removed from soils, and then soils were disaggregated to pass a 4 mm sieve. For analyses of soil properties, dry soil samples were ground and passed through a 2 mm sieve. Soil pH was determined after mixing 1 g of soil in 2.5 ml water for about 5 min, allowed ionic exchange to reach equilibrium prior to measuring pH (Black et al., 1982). Organic matter was determined by loss on ignition. For metal analysis (Ni, Ca, K, P, Mg, Co, Mn and Zn) five grams air dried soil was digested with 10 ml concentrated HNO_3 and heated to near dryness on a hotplate, subsequently dissolved in 20 ml 3 M HCl and heated at mild reflux for 2 h. The residue was filtered and diluted to 50 ml with 0.1 M HCl (Association of Official Analytical Chemists (AOAC) method 3.014 (a), 1984). Metal concentrations were determined using both flame atomic absorption spectrometry (AAS) with deuterium background correction, and inductively coupled plasma atomic emission spectrometry (ICP-AES) using yttrium as an internal standard.

2.2. Bacterial solubilization of metals in soil

We selected nine bacterial isolates for study of their effect on Ni extraction and uptake into *A. murale* grown on low, moderate and Ni-rich soils. These bacteria were originally isolated from the rhizosphere of *A. murale* grown on Oregon Ni-rich serpentine soil (R.A. Abou-Shanab et al., unpublished data). The nine isolates were identified as *Acidovorax avenae* AY512827, *Clavibacter xyli* AY509236, *Microbacterium arabinogalactanolyticum* AY509225, *M. arabinogalactanolyticum* AY509226, *M. oxydans* AY509219, *M. oxydans* AY509221, *M. oxydans* AY509222, *M. oxydans* AY509223 and *Rhizobium galegae* AY509213, based on 96%, 98%, 98%, 97%, 94%, 99%, 99%, 99% and 99% similarity in their 16S rDNA sequence analyses, respectively (Abou-Shanab and van-Berkum, 2003). Specific characteristics of these bacteria include: *Acidovorax avenae* AY512827, *M. arabinogalactanolyticum* AY509225 and *M. oxydans* AY509223 are acid and siderophore producers, nonphosphate solubilizer and can grow on 8 mM $NiCl_2$; *M. oxydans* AY509221, *M. oxydans* AY509222 and *R. galegae* AY509213 are acid producers, nonphosphate solubilizers, nonsiderophore producers, grow on 8 mM $NiCl_2$; *Clavibacter xyli* AY509236, *Microbacterium oxydans* AY509219 and *M. arabinogalactanolyticum* AY509226 are acid producers, phosphate solubilizers, nonsiderophore producers and can grow on 8 mM $NiCl_2$ (Abou-Shanab et al., 2003a).

Bacterial cells were grown overnight in 250-ml Erlenmeyer flasks containing 100 ml of sterilized R2A broth (Reasoner and Geldreich, 1985) on a shaker at 150 rev min^{-1} at 30 °C until late log phase. Bacterial cells were then harvested by centrifugation (12 000*g*, 20 °C, 10 min), and the pellets were washed twice with sterile distilled water. Bacterial suspensions in distilled water were adjusted to an absorbance at 600 nm of 0.5 (equivalent to approximately 7.4×10^8 cfu ml^{-1}). One ml of each bacterial suspension was used as inoculum for 10 g soils in 50 ml Falcon tubes. One ml of deionized sterile water was used for 10 g soils in 50 ml Falcon tubes as the (axenic) control. Soils were incubated at room temperature for 18 d. Extractable metals were measured by shaking soil in Falcon tubes for 2 h in 20 ml of 10 mM $Sr(NO_3)_2$ (Abou-Shanab et al., 2003b). Samples were filtered and acidified with HNO_3 before analysis. Metal concentrations were determined using both AAS and ICP-AES.

2.3. Pot experiment

2.3.1. Bacterial inoculation of A. murale

Bacterial cells were grown in 500-ml Erlenmeyer flasks containing 250 ml of sterilized R2A broth overnight, until late log phase. Bacterial cells were collected as before, washed twice with sterile 0.85% saline (NaCl) solution and

then vortexed to suspend the bacteria. One milliliter of this suspension was added to 4 ml of sterile 0.5% methyl–cellulose solution prepared with 0.85% sterile saline solution to provide a bacterial suspension for inoculation of seeds. Cell densities were adjusted to an absorbance at 600 nm of 0.5 (equivalent to approximately 7.4×10^8 cfu ml^{-1}) and were used for seed inoculation.

Before seeding, *A. murale* seeds were surface-sterilized by shaking in 70% ethanol for 5 min, followed by shaking in 2% sodium hypochlorite for 30 min. Sterile seeds were next washed in five, 5-min rinses with sterile water (Abou-Shanab et al., 2003b). Surface sterilized seeds were soaked in the methylcellulose solution containing each bacterial species. Seeds were soaked in sterile methylcellulose solution as a control. After 20-min soaking time, all seeds were removed from the methylcellulose solution and dried on sterile filter paper in a laminar-flow cabinet (Whiting et al., 2001).

Five hundred grams of low, moderate and Ni-rich soils (Christiana, Chrome and Brockman), respectively, were placed in plastic pots. Seeds coated with bacterial inoculums and axenic seeds (control) were planted into each soil. After 2 weeks, the plants were thinned to four plants per pot. Plants were grown for 2 months in a glasshouse at 25–27 °C and a 16/8-day/night regime. Tap water and nutrient solutions of KNO_3/ $(NH_4)_2$ SO_4 and K_2HPO_4/ KH_2PO_4 were added as needed. All treatments were replicated four times.

2.3.2. Plant harvest and preparation

After 2 months, whole *A. murale* plants were removed from the pots. Shoots and roots were separated. Plant shoots were washed with deionized water, rinsed and dried at 65 °C. Dry plant samples were weighed and ground using a Wiley Mill. Two grams or less were ashed at 480 °C for 16 h. The ash was digested with concentrated HNO_3, dissolved in 3 M HCl, filtered and diluted to 25 ml with 0.1 M HCl (AOAC Official Method of Analysis 3.014 (a), 1984). Metal concentrations were determined using both AAS and ICP-AES using yttrium as an internal standard.

2.4. Statistical analyses

Data were analyzed using SAS version 8.2 (SAS Institute Inc., 1999–2001). Treatment means were separated using the Waller-Duncan *K*-ratio *t*-test after it was determined that there was a significant ($P < 0.05$) treatment effect using the GLM procedure.

3. Results

3.1. Soil physicochemical properties

Soils tested in this study differed in their properties and total metal concentrations (Table 1). Each soil sample exhibited a high concentration in one or more of the metals. The high and medium Ni soils contained large quantities of Ni, Cr, Fe, Co and Mn characteristic of serpentine soils, an appreciable amount of Mg, and little organic matter. The highest Ni concentration of 3590 mg kg^{-1} was found in the high Ni Brockman soil followed by the medium Ni Chrome soil with 930 mg kg^{-1}. In contrast, Ni, Mn, Co and Zn contents of the low Ni soil were low. The soil pH was around 6, and differed slightly between the three soils.

3.2. Effect of bacterial inoculation on metal extractability in Christiana, Chrome and Brockman low, moderate and Ni-rich soils

Eight bacterial isolates significantly increased Ni solubilization in all soils compared with the uninoculated soil. One bacterial isolate, *M. oxydans* AY509223 significantly increased Ni solubilization in high and medium Ni soils but had no effect on Ni extraction from the low Ni soil compared with control. The concentration of extractable Ni was increased from a control of 0.002, 0.57 and 0.95 (in the low, medium and high Ni soils), respectively, to 0.03, 0.68 and 1.08 mg kg^{-1} soil dry matter, respectively, when the soils were inoculated with *M. arabinogalactanolyticum* AY509225 (Fig. 1). None of the nine bacterial isolates had a significant effect on Mg extraction from soils. *M. oxydans* AY509219 had significantly increased Zn solubilization in the low and high Ni soils (data not shown). These results indicate that the activity of the bacteria in the soil would very likely had a significant effect on increasing the mobility of metals in the rhizosphere of the plants in soils.

3.3. Effect of bacterial inoculation on A. murale growth and metal uptake

To determine the effects of rhizobacterial inoculation on *A. murale* growth and metal uptake, uninoculated and inoculated *A. murale* were grown in the three soils. After 2 months, shoots fresh and dry weight of *A. murale* was determined. Fresh and dry weight of *A. murale* uninoculated (control) plants were grown in the low metal soil were

Table 1
Physical and chemical characteristics of soils used

Soil	Organic matter (%)	pH (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	Co (mg/kg)	Mg (mg/kg)	P (mg/kg)	K (mg/kg)	Ca (mg/kg)	Ni (mg/kg)
Low Ni soil	2.8	6.0	20	228	3	161	99	161	624	17
Moderate Ni soil	4.1	6.4	172	1213	90	116	5	67	604	930
High Ni soil	4.0	6.5	100	2133	228	82	3	32	489	3590

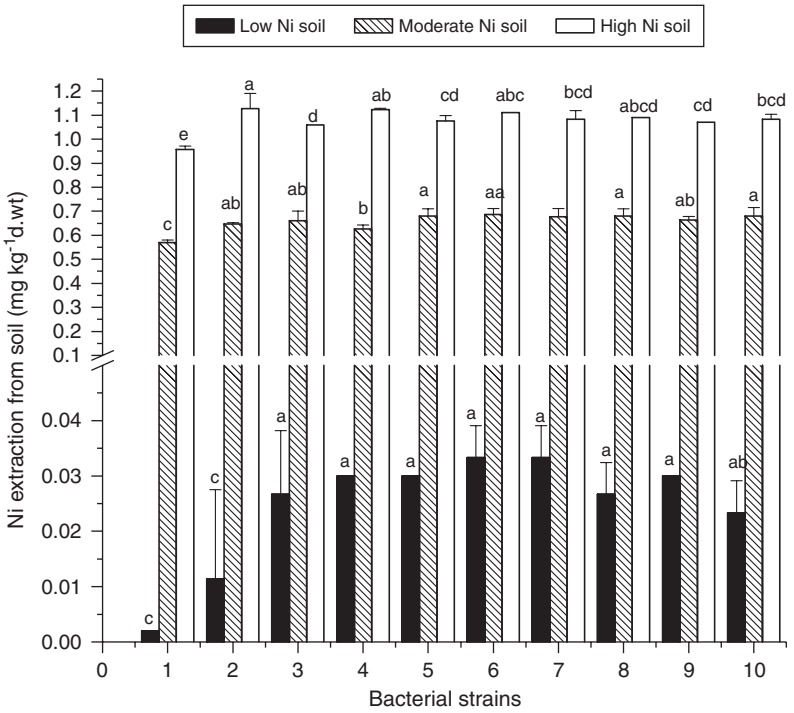

Fig. 1. Effects of inoculation with different bacterial isolates on solubilization in different Ni- contaminated soils, expressed as Ni extraction from soil ($mg\,kg^{-1}$). (1) Control; (2) *Microbacterium oxydans* AY509223; (3) *Rhizobium galegae* AY509213; (4) *M. oxydans* AY509219; (5) *Clavibacter xyli* AY509236; (6) *Acidovorax avenae* AY512827; (7) *M. arabinogalactanolyticum* AY509225; (8) *M. oxydans* AY509222; (9) *M. arabinogalactanolyticum* AY509226 and (10) *M. oxydans* AY509221. Mean values of Ni-extraction with the same letter within the same soil are not significantly different at $P<0.05$. The bar on the top of column is the S.D.

2.32, 2.78-fold and 0.67, 0.69-fold higher than fresh and dry weight of the plants were grown in the high and medium Ni soils, respectively. There were no significant effects of bacterial inoculation on fresh and dry weight of *A. murale* shoots were grown on any of the soils compared with uninoculated soils. Shoot dry and fresh weights of *A. murale* grown in the low metal soil inoculated with *M. oxydans* AY509221 were significantly reduced compared with uninoculated plants (Figs. 2 and 3). Fresh weight of *A. murale* shoots grown in the medium Ni soil inoculated with *M. oxydans* AY509219 was significantly higher compared with uninoculated soil.

To study the effect of bacterial inoculation on Ni accumulation in plants grown in Christiana, Chrome and Brockman soils, nine different bacterial isolates were added individually to surface sterilized *A. murale* seeds at the time of sowing. Bacterial isolates, *M. oxydans* AY509223; *M. oxydans* AY509219; *M. oxydans* AY509222 significantly increased the Ni uptake of *A. murale* grown in the low Ni soil by 36.1%, 32.8%, and 33.1%, respectively, compared with uninoculated seeds (Fig. 4). In the medium Ni soil, Ni-uptake by *A. murale* was significantly increased compared with uninoculated seeds by 39.3%, 32.6%, 25.6%, respectively, as a result of inoculation with *M. oxydans* AY509223, *M. oxydans* AY509219 and *R. galegae* AY509-213, respectively. *M. arabinogalactanolyticum* AY509225; *M. oxydans* AY509222 and *M. oxydans* AY509223 significantly increased the Ni uptake of *A. murale* grown in the high Ni soil by 31.9, 29.4, and 27.7%, respectively, compared with uninoculated seeds. *M. oxydans* AY509223 significantly increased the Ni uptake of *A. murale* grown in all soils by 36.1%, 39.3%, and 27.7%, respectively, compared with uninoculated seeds (Fig. 4). *M. oxydans* AY509223 increased foliar Ni from control concentrations of 82.9, 261.3 and $2829.3\,mg\,kg^{-1}$ to 129.7, 430.7 and $3914.3\,mg\,kg^{-1}$, respectively. It was also observed that the highest Ni accumulation of 4154 and $4008\,mg\,kg^{-1}$ was observed in the shoot of *A. murale* grown in the high Ni soil inoculated with *M. arabinogalactanolyticum* AY509225 and *M. oxydans* AY509222, respectively. This demonstrates that plant bioavailability of Ni was affected in a way that did not always affect extraction with a weak acid. There were no significant effects on shoot Ca, Mg, and Mn when bacteria of each isolates were tested (data not shown).

4. Discussion

Total metal content is important because it determines the size of the metal pool in the soil and thus the potential for metal uptake (Ibekwe et al., 1995). Serpentine (ultramafic) outcrops are distributed all over the world and are characterized by high levels of Co, Cr, and especially Ni (Brooks, 1987; Baker and Brooks, 1989; Robinson et al., 1996). The vegetation adapted to survive in these soils can include the so-called nickel hyperaccumulating plants

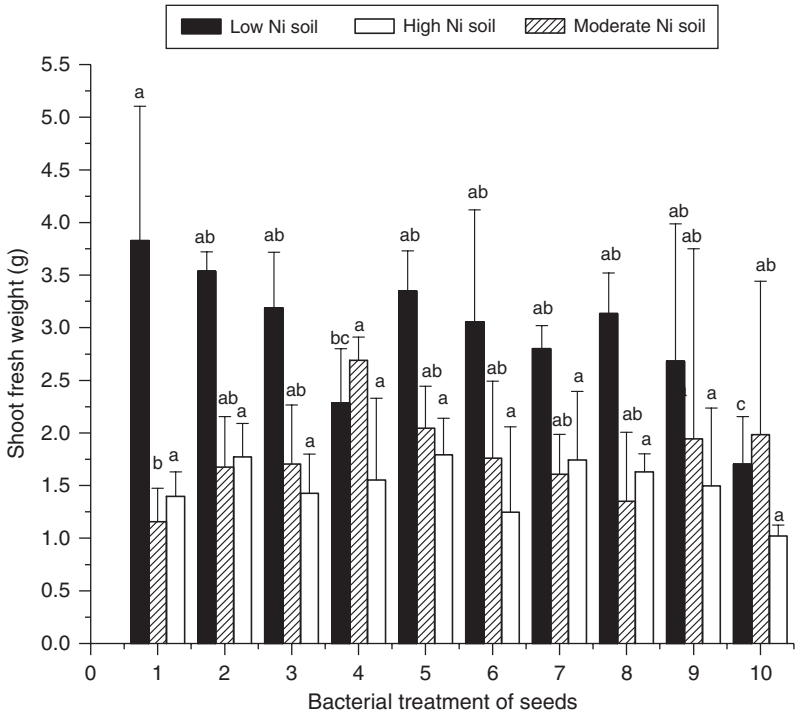

Fig. 2. Effects of inoculation with different bacterial isolates on shoots fresh weight *A. murale* seedling grown in different Ni-contaminated soils. (1) Control; (2) *Microbacterium oxydans* AY509223; (3) *Rhizobium galegae* AY509213; (4) *M. oxydans* AY509219; (5) *Clavibacter xyli* AY509236; (6) *Acidovorax avenae* AY512827; (7) *M. arabinogalactanolyticum* AY509225; (8) *M. oxydans* AY509222; (9) *Microbacterium arabinogalactanolyticum* AY509226 and (10) *M. oxydans* AY509221. Mean values of shoot fresh weight with the same letter within the same soil are not significantly different at $P<0.05$. The bar on the top of column is the S.D.

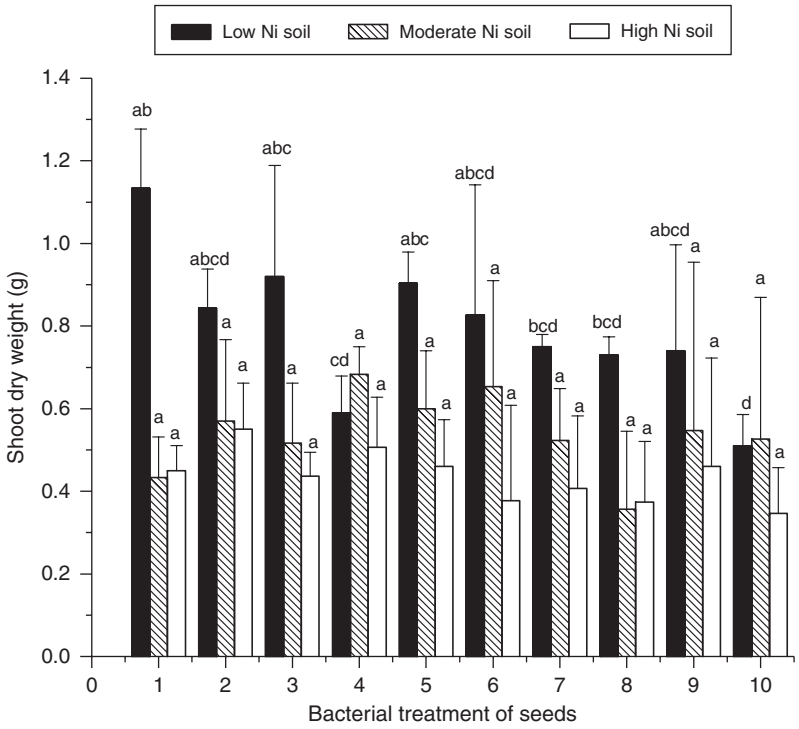

Fig. 3. Effects of inoculation with different bacterial isolates on shoots dry weight *A. murale* seedling grown in different Ni-contaminated soils. (1) Control; (2) *Microbacterium oxydans* AY509223; (3) *Rhizobium galegae* AY509213; (4) *M. oxydans* AY509219; (5) *Clavibacter xyli* AY509236; (6) *Acidovorax avenae* AY512827; (7) *M. arabinogalactanolyticum* AY509225; (8) *M. oxydans* AY509222; (9) *M. arabinogalactanolyticum* AY509226 and (10) *M. oxydans* AY509221. Mean values of shoot dry weight with the same letter within the same soil are not significantly different at $P<0.05$. The bar on the top of column is the S.D.

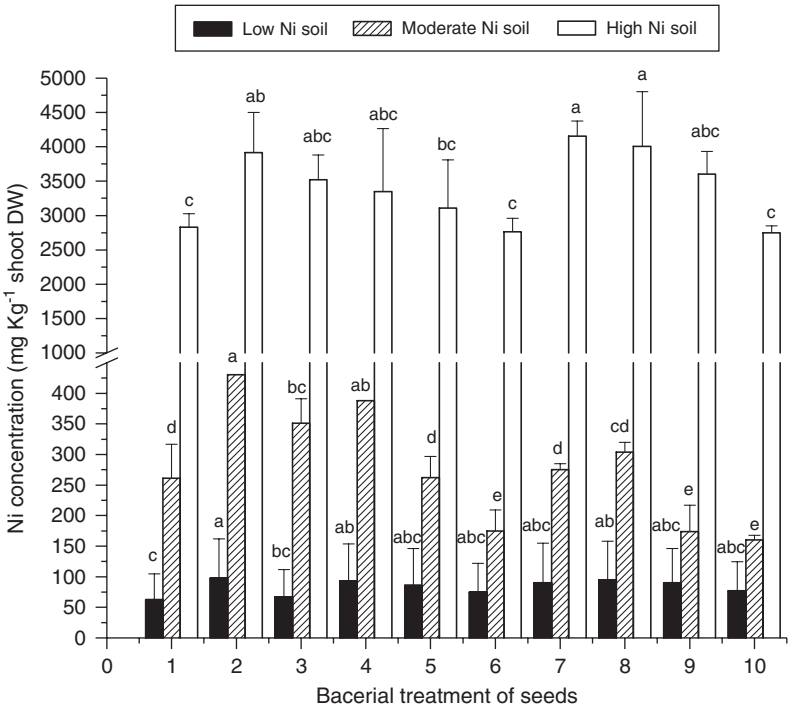

Fig. 4. Effects of inoculation with different bacterial isolates on Ni uptake by *A. murale* seedling, expressed as plant shoot Ni concentration. Mean values of Ni-uptake with the same letter are not significantly different at $P<0.05$. (1) Control; (2) *Microbacterium oxydans* AY509223; (3) *Rhizobium galegae* AY509213; (4) *M. oxydans* AY509219; (5) *Clavibacter xyli* AY509236; (6) *Acidovorax avenae* AY512827; (7) *M. arabinogalactanolyticum* AY509225; (8) *M. oxydans* AY509222; (9) *M. arabinogalactanolyticum* AY509226 and (10) *M. oxydans* AY509221. Mean values of Ni-uptake with the same letter within the same soil are not significantly different at $P<0.05$. The bar on the top of column is the S.D.

(plants which accumulate more than 1000 μg Ni g^{-1} dry weight shoot tissue) (Baker, 1981) that concentrate metal in stems and leaves to levels higher than the substrate concentration and far in excess than any physiological requirement. Among the best-known hyperaccumulators, *A. murale* is able to colonize serpentine soils and accumulate nickel in excess of 2% (W/W) of shoot dry matter (Reeves and Baker, 2000; Li et al., 2004; Chaney et al., 2005).

Microorganisms are ubiquitous in soils to which hyperaccumulators are native, even in those soils containing high concentrations of metals (Schlegel et al., 1991; Ghaderian et al., 2000). Soil microorganisms can affect trace metal mobility and availability to the plant, they can produce iron chelators and siderophores for ensuring iron availability, reduce soil pH, and or solubilize metal-phosphates. Microbes influence root parameters, such as root morphology and growth. An increase in root exudation of organic solutes could affect the rate of phytosiderophore release. In turn, rhizosphere microorganisms may interact symbiotically with roots to enhance the potential for metal uptake (Burd et al., 2000; Guan et al., 2001). Abou-Shanab et al. (2003b) reported that concentration of extractable Ni was increased from a high Ni soil of 2.2 to 2.6 mg kg^{-1} when the soil was inoculated with *M. arabinogalactanolyticum* AY509224. Acid, siderophore production and phosphate solubilization by the bacterial isolates facilitated Ni solubility in the nonsterile, Ni-rich soils. Siderophore production can also be stimulated by the presence of heavy metals (van der Lelie et al., 1999) and they possibly affect bioavailability of a variety of metals. For instance, it was reported that *Azobacter vinelandii*, siderophore production is increased in the presence of Zn(II) (Huyer and Page, 1988). Iron chelating hydroxamic acid production in *Bacillus megaterium* is increased by exposure to Cu, Cr, Cd, Zn, and Al were found to increase siderophore production in *Pseudomonas aeruginosa* (Gilis, 1993). The same effect was found for Zn and Al in *P. fluorescens* ATCC17400 (Gilis, 1993).

Clearly, despite the fact that *Alyssum* hyperaccumulates Ni even without rhizobacteria inoculation, bacteria can appreciably increase the accumulation of Ni for hyperaccumulation from soils with a high proportion of non-soluble Ni. This study indicates that bacteria facilitated the release of Ni from the non-soluble phases in the soil, thus enhancing the availability of Ni to *A. murale*. A possible explanation might be acid, siderophore production and phosphate solubilization. These effects of inoculation were reported also by Whiting et al. (2001), who found that the addition of a mixed inoculum of *M. saperdae*, *P. monteilii*, and *E. cancerogenes* to surface sterilized seeds of *Thalaspi caerulescens* sown in autoclaved soil increased the Zn concentration in shoots 2-fold compared with non-inoculated controls; the total accumulation of Zn was enhanced 4-fold.

Although many soil bacteria are tolerant to heavy metals and play important roles in mobilization or immobilization

of heavy metals (Gadd, 1990), only a few attempts have been made to study the rhizosphere bacteria of metal accumulating and hyperaccumulating plants and their role in the tolerance to and uptake of heavy metals by the plants. A high proportion of metal resistant bacteria persist in the rhizosphere of the hyperaccumulators *T. caerulescens* (Delorme et al., 2001) and *A. bertolonii* (Mengonii et al., 2001) or *A. murale* (Abou-Shanab et al., 2003a) grown in soil contaminated with Zn and Ni or Ni, respectively. The presence of rhizosphere bacteria increased concentrations of Zn (Whiting et al., 2001), Ni (Abou-Shanab et al., 2003b) and Se (De-Souza et al., 1999) in *T. caerulescens*, *A. murale* and *B. juncea*, respectively. Inoculation of Indian mustard and canola (*Brassica campestris*) seeds with the plant growth-promoting rhizobacteria (PGPR) strain *Kluyvera ascorbata* SUD165, which produces siderophores and contains the enzyme 1-amino-cyclopropane-1-carboxylate (ACC) deaminase, protected the plant against Ni, Pb and Zn toxicity (Burd et al., 1998). Inoculation of *B. napus* with metal resistant PGPR containing ACC deaminase stimulated growth of plants cultivated in Cd contaminated soil (Belimov et al., 2001).

Enhanced uptake of Ni by *A. murale* is valuable to the overall economics of phytoextraction of Ni form soils. As the technology of Ni 'phytomining' matures and is commercially developed, even small increases in Ni uptake can have very significant impacts on profitability. Thus, if findings of this work translate into practical field application, revenue generated during phytomining can potentially be increased substantially.

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